



RESEARCH ARTICLE

REVISED The Discrepancy between Clove and Non-Clove Cigarette Smoke-Promoted *Candida albicans* Biofilm Formation with Precoating RNA-aptamer [version 3; peer review: 2 approved]

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Abstract

This study explores the influence of precoating aptamer (Ca-apt1) on *C. albicans* viability while the fungus was growing in the presence of exposing condensed cigarette smoke (CSC), prepared from clove (CCSC) and non-clove (NCSC) cigarettes, for 48 h. Using qPCR, we found that mRNA expression of adhesion-associated genes (*ALS3* and *HWP1*) was impaired by precoating *C. albicans* yeast cells with the aptamer. Conversely, the gene transcription was upregulated when aptamer-uncoated yeast was pre-treated with either CSC. In addition, by analysing the result of MTT ([3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay, we found that the presence of added CCSC or NCSC in growth medium for 48 h was significantly enhanced *C. albicans* biofilm development. However, the presence of precoated aptamer was significantly impaired biofilm development accelerated by the NCSC. The inhibitory effect of the Ca-apt1 was not dependent on the precoated aptamer (1ng/μL and 10 ng/μL). Interestingly, we noted that the enhancer effect of treated CCSC was no longer effective when the yeast had been precoated with 10 ng/μL aptamer tested. Additionally, light microscopy analysis revealed that precoating aptamer alleviates morphological changes of *C. albicans* (from yeast to hypha formation) that are enhanced by adding CCSC or NCSC in the growth medium. In conclusion, these results suggest that administration on Ca-apt1 exhibits a significant protective effect on CSC-induced biofilm formation by *C. albicans*.

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Keywords

Cigarette Smoke, *Candida albicans*, Biofilm , RNA-aptamer, ALS3 and HWP1.

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REVISED Amendments from Version 2

In this revised version, we made corrections to some typos. Also, as suggested by the reviewer, the concentrations of the used aptamer have been changed to 1 ng/ μ L and 10 ng/ μ L instead of 1% and 10%, respectively.

Any further responses from the reviewers can be found at the end of the article

Introduction

Candida albicans is a normally harmless inhabitant of the oral cavity. However, unlike other fungal pathogens that exist primarily in either yeast or hyphal forms, *C. albicans* is an opportunistic pathogen. The fungus's behaviour correlates with its ability to grow in distinct morphogenic states, including budding yeast or blastospores, pseudo hyphae, and true hyphae¹⁻³. This morphogenic transition from yeast to hypha form is important for the pathogenesis of *C. albicans*, is dependent on how the fungus cell responds to the environmental cues⁴. As shown in the literature, cigarette smoke is one factor that can aid and accelerate this transformation⁵⁻⁷, and there is a clear association between oral candidiasis and smoking habit⁸. In this regard, the presence of nicotine in cigarette smoke promotes *C. albicans* growth rate and adhesion, in which, a higher level of expression of genes related to adherence, such as *ALS3* and *HWP1* have been reported⁹.

Smoking is a social habit in many countries¹⁰, and it is an important public health problem, including in Indonesia¹¹. However, despite the extensive research exploring the deleterious role of a smoking habit on oral microorganisms, there is little information about the effect of non-conventional tobacco, i.e., clove cigarettes, on the virulence attributes of *C. albicans*. By searching the literature, we found that, like conventional cigarettes, the clove cigarette, commonly known as kretek, is the most popular¹² in Indonesia. It is also a source of numerous toxicants, and they have a potential implication in the oral ecosystem¹³, which may also influence oral *Candida* pathogenicity¹⁴. The unique aspect of kretek is the dried clove buds it contains¹⁵ which have never been identified in a conventional cigarette. The involvement of clove cigarette smoke condensate (CCSC) on the biofilm formation of *C. albicans* remains obscure. Given that cigarette smoke contains many toxicants¹³, it is important to explore the involvement of CCSC on the growth and morphogenesis of *C. albicans*, as this fungus is the most implicated oral pathogen in the clinical setting.

During the last several years, aptamers, either single strand DNA or RNA, and against different microorganism species, have become the focus of growing interest. In an earlier study, we reported an anti-*Candida* activity of an RNA-aptamer (Ca-apt1) against the fungus while growing as biofilm¹⁶. Here, we evaluated the aptamer's beneficial properties against *C. albicans* biofilm formation induced by cigarette smoke. Particularly, the aptamer's ability to restrict the transition of fungus phenotype from yeast to hyphal form.

Methods

C. albicans and RNA Aptamer used

In this study, we used a clinical isolate that we collected previously from the denture surface of denture wearer subject¹⁷ and selected by using CHROMAgar¹⁸, while the reference strain (*C. albicans* ATCC 10231), which was used as a targeted aptamer ligand¹⁶, was needed for the evaluation and validation of this experimental study. Briefly, all microorganisms were taken from stock cultures frozen in 15% glycerol at -80°C and sub-cultured onto yeast peptone agar plates (1% yeast extract, 2% peptone, 2% glucose; YPD) or when indicated in yeast nitrogen base medium, supplemented with 50 mM glucose (YNB).

The synthetic oligonucleotide used in this study was RNA-aptamer (Ca-apt 1) that was obtained from the systemic evolution of ligands by exponential enrichment (SELEX) method¹⁶.

Preparation of cigarette smoke condensates

In this study, condensed smoke cigarettes (CSC) were generated from Indonesia's non-filtered clove cigarette (kretek) and imported non-clove cigarettes (Figure 1) that we purchased from a local tobacco outlet. The CSC, either from a clove cigarette (CCSC) or a non-clove cigarette (NCSC), was prepared by smoking five cigarettes that were smoked to an in-house smoking device⁵ and concentrated in 100 mL of 0.09% Sodium Chloride solution. The resulting condensate smoke solution was further sterilized by filtration through a Millipore filter (0.22 μ m). The influence of CCSC/ NCSC-containing YNB (pH was adjusted to 7.2) on the viability of aptamer-precoated *C. albicans* in the preformed biofilm was assessed colorimetric (MTT) assay and kept at 4°C until use.

Analysis of mRNA expression of *ALS3* and *HWP1* and microscopic image in *C. albicans* with and without precoating aptamer

To determine that the inhibition effect of the tested aptamer in the early biofilm development stage was independent from the biofilm biomass maturation, we separated biofilm development into adhesion and growth phases. Initially, by analysing the mRNA expression of adhesion-associated genes (*ALS3* and *HWP1*), we wanted to confirm if the inhibition event was due to the lessened ability of *C. albicans* to switch its phenotype from yeast to hypha form. To do this, the test aptamer was first preincubated with *C. albicans* yeast cells before the fungus was inoculated into microplate wells. Further, we did an analysis of mRNA expression of adhesion-associated genes (*ALS3* and *HWP1*) after 90 min incubation time. The transcription level of these genes was also measured after *C. albicans* was exposed with either cigarette smoke, but without precoating aptamer. This was done to evaluate CSC's involvement in the transition *C. albicans* morphogenetic, from yeast or blastospore to hyphae form. All the procedures used as described in a previous study¹⁷. Briefly, RNA isolation, purification, and reverse transcription of cDNA were conducted using TRIzol™ Reagent (Invitrogen Life Technologies, Carlsbad, California, United States) followed by reverse transcription using the



Figure 1. Cigarettes used to prepare condensate cigarette smoke. A and B are Clove and non-clove cigarette, respectively.

TaqMan® Reverse Transcription Reagents (Applied Biosystems, Waltham, Massachusetts, USA). The resulting cDNA (1 µg) was amplified by qPCR with specific primers used in our previous study¹⁹. The qPCR analysis was performed in ABI StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) with Platinum™ SYBR™ Green qPCR SuperMix-UDG (Invitrogen). The qPCR cycling conditions consisted of a 10-minute initial denaturation at 95°C followed by 40 PCR cycles of 15 seconds at 95°C and one minute at 60°C. The formula of fold change $2^{-\Delta\Delta Ct}$ was used to calculate the relative mRNA expression, which was compared with that of the house-keeping gene, 18S rRNA. The mRNAs gene (*ALS3* and *HWP1*) expressed by *C. albicans* without bound aptamer or without CSC exposure were used as a control, set at one.

Biofilm formation of *C. albicans*

To test the effectivity of CSC, with and without precoating aptamer, on *C. albicans* biofilm formation, we used a biofilm assay that was performed as previously described¹⁹. Briefly, 100 µL containing 1.8×10^5 yeast cells of *C. albicans* (counted by using a haemocytometer), from overnight culture in YPD broth was aliquot into 96-well microtiter plates containing mixture of 70% of fresh yeast nitrogen base (YNB; sigma-Aldrich) and 30% condensate smoke (vol/vol). The pH of the mixture was adjusted to neutrality (7.0) using 1M NaOH. This was done to define that the pH of the mixture does not play a role in modulating biofilm formation.

The aptamer was prepared by separating it in two different concentrations (1ng/µL and 10 ng/µL) in buffer¹⁶, prior to being added into separate wells, and the plates were incubated at 37°C in 5% CO₂ in air for 90 min with gentle shaking. *Candida albicans* biofilm with mix medium added instead of aptamer was used as a negative control. To promote biofilm formation, the adhered cells were washed twice with sterile Phosphate Buffer Saline (PBS), *C. albicans* were further grown in a medium (150 µL) containing growth medium without aptamer. The culture period was further lengthened to 48-h. The extent of biofilm formation estimated using the semi-quantitative MTT (3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) reagent (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was measured spectrophotometrically at 450 nm with 620 nm as the reference wavelength for this assay. The results

expressed as OD_{450/620}, and were correlated with cellular metabolic activities within the biofilm. The assay was done in triplicate, repeated two times independently. Moreover, the percentage of fungal damage was calculated based on data obtained in MTT assay, using the formula: $100X [(1 - OD_{CandidaCSC+apt} / OD_{Candida\ alone})]$. Biofilm formation on the bottom of microtiter well plates was qualitatively observed using an inverted light microscope.

Analysis of microscopy image

Following incubation for 90 min or 48-h, *C. albicans* cultures were observed microscopically and its morphology images were analysed qualitatively.

Statistical analysis

Statistical analysis was performed with GraphPad Prism™ (version 9.00) software (GraphPad Software, Inc., San Diego, California, USA). The two-way analysis of variance (Two-way ANOVA) with Geisser-Greenhouse correction was used to verify the significant level of response between and within groups comparison. Each experiment was carried out in triplicate wells and repeated at least twice, independently. A $P < 0.05$ value was considered statistically significant.

Consent statement

Written informed consent was obtained from participants for use of their data.

Ethical approval

The samples were collected, after informed consent was obtained from all the participants¹⁷, in accordance with the approved protocol of the Bioethics Committee of the Faculty of Dentistry, Universitas Indonesia (protocol number 020950818). The protocol conformed to the criteria of the Helsinki Declaration and the good clinical practical guidelines of the International Council on Harmonization.

Results

Evaluation of the mRNA levels of *ALS3* and *HWP1* in pre-coating aptamer *C. albicans*

To understand the underlying relationship between precoating aptamer (Ca-apt1) and *C. albicans* morphogenesis at molecular level, we firstly compared the expression profile of two

adhesion-associated genes (*ALS3* and *HWP1*). To do this, the test aptamer was preincubated with *C. albicans* yeast cells before the biofilm was allowed to form. Further, we combined quantitative analysis of mRNA expression of these genes and qualitative microscopic images to describe the adhesion event. The result showed, that after 90 min, the expression of *ALS3* by the adherence cells were proportionally reduced by $\approx 8\%$ and $\approx 4\%$, for aptamer concentration 1 ng/ μ L and 10 ng/ μ L, respectively. A similar trend in the expression of adhesion-associated genes was noted in either *C. albicans* strain used. We noted that the reduction of *HWP1* expression was higher than *ALS3* under different concentrations of Ca-apt1. In either *C. albicans* strain, this gene downregulated was $> 50\%$ (Figure 2A and B). Further, the microscopic images show that at the attachment phase (90 min), the biofilm consisted of both yeast cells and blastospore that adhered to the surface, and those cells adhered to other cells attached to the surface (Figure 2C–E).

CCSC and NCSC promoted the transcription level of *ALS3* and *HWP1* by *C. albicans*

Next, we tested the mRNA expression of *ALS3* and *HWP1* on the biofilm formation promoted by either cigarette smoke condensate (CSC) after 48-h incubation time without precoating aptamer. As shown in Figure 3A and B, by comparing CCSC and NCSC, we observed that *Candida* adhesion was due to the presence of CCSC that subsequently increased the transcription level of hypha-associated gene (*HWP1*), compared to the biofilm induced by NCSC-added growth medium ($p < 0.05$). For the adhesion-related gene (*ALS3*), the transcription level was comparable when induced by either CCSC or NCSC-treated growth medium or growth medium only (control/ unexposed *C. albicans*). We noted that both *C. albicans* strain (clinical isolate and ATCC) showed a similar pattern. Figure 3C–E show the result provided by the microscopic analysis. After a 48-h time period, the hyphal form was more abundant in CCSC-treated growth medium than those in NCSC-treated biofilm.

Precoated RNA aptamer inhibits *C. albicans* biofilm formation

Next, we tested the impact of different concentrations of precoating aptamer (1ng/ μ L and 10 ng/ μ L) on *C. albicans* biofilm formation. For this, biofilms were developed in CCSC/NCSC-containing YNB (pH was adjusted to 7.2), and the quantification of biofilm cell (MTT assay) was evaluated after the biofilms reach the maturation time (48-h). This method was done, because we wanted to determine whether the precoating aptamer could adversely affect *C. albicans* biofilm formation after treating with the biofilm enhancers (CCSC or NCSC). Our data found that in comparison to the control, 1ng/ μ L aptamer concentration of precoated yeast cells was sufficient to reduce biofilm cell, by about 10%, and 40 %, as observed in CCSC and NCSC-treated biofilm, respectively. At this maturation state, the viable cells detected within this biofilm were significantly reduced ($p < 0.05$), because of the increased aptamer concentration by 10% in the pre-adherence phase of the biofilm (Figure 4A–B). Further, the effect of CCSC or NCSC on the

transition of yeast to hypha morphology in pre-adhered cells was visualized microscopically. We observed that when the fungus cells were precoated with aptamer without exposed with CCSC or NCSC and incubated for 48-h, the pre-formed biofilms were dominated by blastospores, as compared to the control cells (uncoated cells), which showed that the adhered cells were dominated by dense hyphae form (Figure 5 A–B). This qualitative effect was also dependent on the concentration of the aptamer tested (Figure 5C–F).

Discussion

The current study attempted to get further insight into clove cigarettes' effect on non-mammalian eukaryotes cells, taking the fungus *C. albicans* as a model system. Initially, as a means to determine that the restriction effect of *C. albicans* to switch its phenotype from yeast to hypha form was due to the presence of the precoating aptamer on yeast, we separated biofilm development into adhesion and growth phases. The result of the transcription assay during the initial stage of biofilm formation indicated that the aptamer involvement in reducing the expression of adhesion and hypha-associated gene (*ALS3* and *HWP3*)²⁰, and the transcription profile was in line with the increased concentration of the aptamer, but not on *C. albicans* strains. This result suggests that the aptamer (Ca-apt1) might bind to a common chemical structure on the fungal yeast cell, probably glucans and chitins²¹ that form the basic cell wall scaffold²². We presume that this aptamer may interfere with the adherence mechanism where these molecules are involved²².

Our study is different from most *in vitro* studies, which use an antibody that recognizes cell-wall proteins, that are commonly related to the host immunological status^{23,24}. In this study, the precoating aptamer (Ca-apt1) does not directly recognize the cell wall proteins as an antibody would. In this way, we assumed that based on the *ALS3/HWP1* profiles explained above, the obvious virulence factors (morphology transformation from yeast to hypha) were more likely to be the main effect of precoating Ca-apt1. This assumption was supported by microscopic observation that at the attachment phase (90 min time point) the morphology of *C. albicans* consisted of both yeast cells and blastospore only. This result confirmed the transcription assay that the aptamer involvement in reducing adhesion and hypha-associated gene (*ALS3* and *HWP3*)²⁰ was in line with the increased concentration, but not on *C. albicans* strains. Likewise, this qualitative data exhibited a significant potentiate of Ca-apt1 in modulating biofilm-associated genes, as well as *C. albicans* morphogenesis, at the early step of biofilm formation.

Although the pattern was similar, in general, the biofilm formation (90 min time point) treated with CCSC resulted in a higher viable cell than its NCSC counterpart, as we observed that after being treated for 48-h by CCSC or NCSC. Different cellular response of *C. albicans* was noted, both by analysing the gene (*ALS3* and *HWP1*) profiles and observing microscopically. At this maturation biofilm state, the induction of hypha-associated gene (*HWP1*) was significantly different, but not for the gene's adhesion (*ALS3*). We noted that the

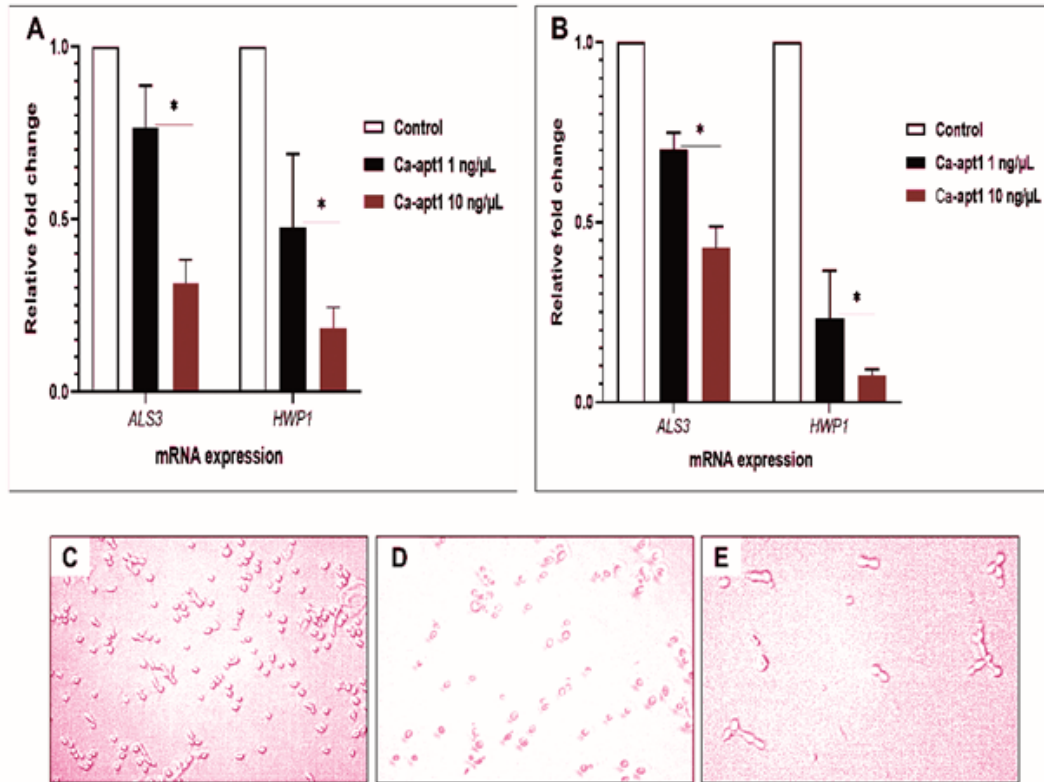


Figure 2. Induction of selected biofilm-associated genes by *C. albicans* following precoating with different concentrations of Ca-apt1. The upper panel shows the effect of precoating aptamer on *ALS3* and *HWP1* expressed by *C. albicans* in preformed biofilm (90 min incubation time) were analysed by qPCR. The mRNA expression levels were normalized relative to the control (yeast cells without aptamer precoating), which was set to one for each gene to determine the fold change in expression of genes in *C. albicans* clinical isolate (A) and the ATCC 10231 (B). The results are expressed as the mean and standard deviation (SD) of triplicate experiments and repeated two times independently. *Significantly higher downregulation in the expression of mRNA ($P < 0.05$) in the presence of precoating aptamer. The lower panel is representative of micrograph of *C. albicans* yeast cell (that only shown in clinical isolate) without precoating aptamer (C), and the reduced germinated cells after incubating for 90 min with precoating aptamer 1 ng/μL (D) and 10 ng/μL (E). All images were captured by using light microscopic at X 200 magnification.

transcription level of *HWP1* by either *C. albicans* strain was higher than *ALS3* only when the preformed biofilm was treated with CCSC. This result indicates that unlike *ALS3*, the transcription level of *HWP1* was dependent on the source of the condensate smoke used. The effect of condensate smoke on *C. albicans* cells viability in biofilm development does not relate to *C. albicans* strain. We assumed that different tobacco components, specifically the principal component alkaloid of tobacco (nicotine), between kretek and conventional cigarettes^{25,26}, may have a different effect on *C. albicans* when the fungus was growing in distinct environmental conditions that trigger the hyphal growth. Additionally, the *HWP1* product (Hwp1) is a hypha-association protein commonly expressed on germ tube^{27,28}. Indeed, this result can be considered after 90 min, when germ tube was induced²⁹. The upregulation of *HWP1* when the biofilm is maturing (48-h) is a survival pathway used by *C. albicans* to resist, or be tolerant against, the effect of chemical toxic-containing CCSC, by modulating the hypha form.

It has been reported that, like NCSC, CCSC comprises a high number of dangerous alkaloid chemical compounds, the most abundant of these being nicotine²⁵. A previous report also showed that the CCSC and NCSC used in this study deliver significant quantities of carbon monoxide (CO), tar and presumably other toxic components of tobacco smoke. Their smoke chemistry were found to be similar³⁰. The other chemical compound in the gas phase of cigarette smoke is nitrogen (N₂)³¹. The nicotine in smoke yields from CCSC is equally delivered as by NCSC³². However, the nitrogen content in CCSC is lower than found in conventional cigarettes/NCSC³⁰. The most distinguished particle-phase, which is only found in CCSC, is eugenol¹⁵. Eugenol is a phenylpropanoid compound reported to have antimicrobial activity against planktonic cells of *C. albicans* and sessile cells within *C. albicans* biofilms³³.

In contrast to the eugenol, the presence of nicotine in cigarette smoke promotes a high level of adhesins leading to the

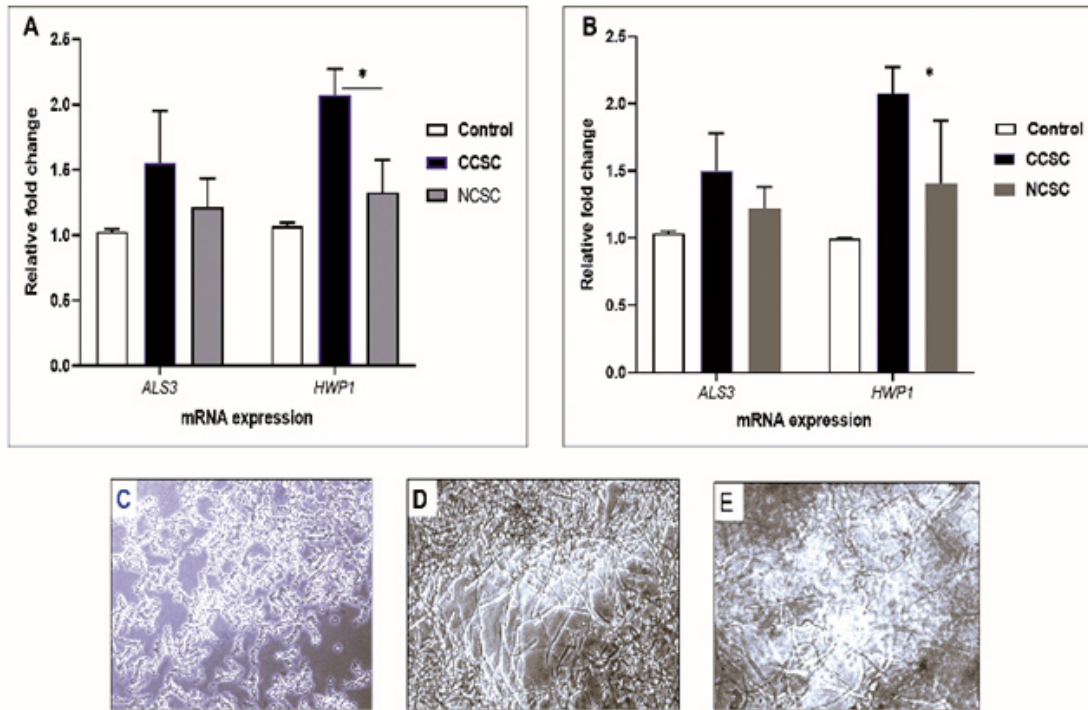


Figure 3. Effect of cigarette smoke condensate on the transcription level of selected *C. albicans* biofilm-associated genes.

The qPCR analysis of *ALS3* and *HWP1* genes is shown after the preformed biofilm (90 min incubation) was treated by growth medium supplemented with different CSC (CCSC or NCSC). The mRNA level of each gene was normalized to that of 18S rRNA, while the expression level of the control (untreated biofilm) was set to one for each gene to determine the fold change in the expression of each targeted gene in clinical isolate (A) and the ATCC strain (B) of *C. albicans*. *Significantly higher upregulation of mRNA expression of *HWP1* induced by CCSC than NCSC ($P < 0.05$). Data are expressed as the mean and standard deviation (SD) of triplicates from two separate experiments. The CCSC and NCSC are clove and non-clove cigarettes smoke condensate, respectively. The lower panel shows the 48-h biofilm formation that only shown in clinical isolate; the control (C) and those biofilms treated with CCSC (D) and NCSC (E), respectively. All the biofilms are visualized by using light microscopic images at X 200 magnification.

increased adherence of *C. albicans*³⁴ on a solid surface, as found in this study. Hence, it has the potential to increase biofilm formation by *C. albicans*. Considering that eugenol impairs the growth of *C. albicans*, we assume that under the experimental condition set in this study, *C. albicans* sensed eugenol in CCSC, and the presence of nitrogen helped it to grow. Since we grew the fungus in a hypha conditioning medium, the result obtained in this study indicates that by comparing with NCSC, the lower nitrogen in CCSC is the likely reason for the augmentation of *C. albicans* biofilm formation^{9,35} enhanced by the CCSC. As we evaluated microscopically, more hypha in 48-h biofilm was observed in response to the presence of CCSC than NCSC.

As shown in the literature, cigarette smoke exposure (CSC) has a potential adverse health effect on the oral ecosystem³⁶, and the growth rate of *C. albicans* increases when the fungus is grown in the presence of non-clove cigarette smoke condensate/NCSC⁵. Indeed, cigarette smoke is an important predisposing factor for oral candidiasis³⁵. Although kretek/clove cigarette smoke condensate (CCSC) and NCSC contains many toxicants, they have some different chemical constituents^{30–32},

which may lead to the differences in *Candida* cell behaviour that we observed in this study.

We further address whether the precoating aptamer could adversely affect the preformed biofilm formation's viability when treated with CCSC or NCSC. The experiment was done to confirm that, on the one hand, experimental *C. albicans* biofilm formation promoted by cigarette smoke (CCSC and NCSC) could be induced. On the other, that the precoating aptamer is needed for the success of this model. In this way, the discrepancy between CCSC and NCSC on *C. albicans* susceptibility was measured using a MTT reduction assay. This is a reliable test for an indirect method to quantify biofilm cell numbers^{37,38}. It has been demonstrated that when growing as a biofilm, the metabolic activity of *C. albicans* increases over time³⁹. However, another factor (the aptamer) was added in the current study. Here, we observed that the control group (*C. albicans* of untreated condensate cigarette smoke/ CSC, without precoating aptamer (Ca-apt1) remained growing as a biofilm throughout the experimental time period set in this study. This result may suggest that *C. albicans* used the tobacco compound as a

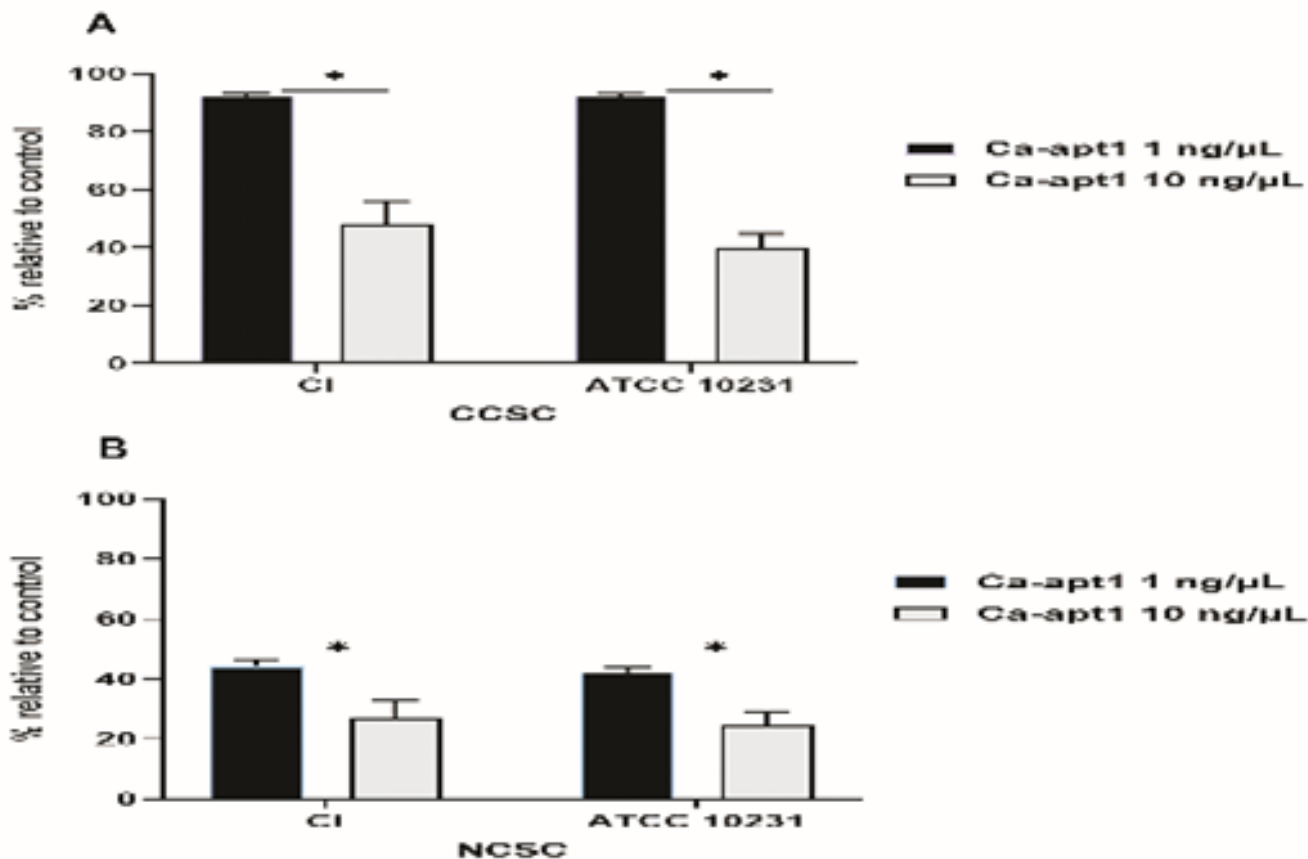


Figure 4. Effect of condensate smoke on preformed biofilm cells. The effect of CCSC (A) and NCSC (B) exposure on biofilm formation of *C. albicans*, which had been pre-coated with different concentration of Ca-apt1, evaluated after incubation for 48-h. The relative biofilm formation compared to biofilm without the pre-coating aptamer (control) was calculated. Data represent the mean and standard deviation (SD) of three biofilms grown on two separate occasions. Asterisks denote statistically significant differences between the effect of cigarette smoke condensate determined by MTT assay, $p < 0.05$. The CCSC and NCSC are clove and non-clove cigarette smoke condensate, respectively.

nutritional source, as aromatic hydrocarbons in cigarette smoke can be converted by the fungus³⁵.

Further, we found that after treating with NCSC for 48-h, the lowest concentration of tested aptamer (1ng/μL) was enough to reduce the cell viability > 50%. This indicates that the effectiveness of NCSC as an accelerator of biofilm formation⁵ was significantly suppressed. Hence, it affirms the anti-adhesion effect of the aptamer on biofilm development accelerated by conventional cigarette smoke^{5,7}. Surprisingly, aptamer-precoated *C. albicans* behaves differently upon exposure to growth medium-containing CCSC. We noted that at one ng/μL of the aptamer, significant cell growth changes were not observed compared to the control (uncoated aptamer). However, at 10 ng/μL, the aptamer could inhibit the biofilm cell growth until *C. albicans* reached a steady state at a 48-h time period, more or less at a similar rate to those cells exposed by NCSC. The result was supported by the light microscopy data, in which we observed that

when the fungus was exposed with the minimal concentration (1 ng/μL) of Ca-apt1, the composition of 48-h-old biofilms consisted of pseudo hypha, almost similar to the control (*C. albicans* without pre-coated aptamer). We also observed that when the concentration of the tested aptamer was increased by 10-fold, it prevented the cell's viability and successful germination of the adherence cells, resulting in scant biofilms, and more yeast cells were observed microscopically in treated groups (CCSC and NCSC exposure). For NCSC, we observed that regardless of the tested aptamer's concentration in pre-coating step, the NCSC-treated biofilms were predominantly in the blastospore or yeast form compared to the control. After 48-h exposure, many of these biofilm cells were unbudded and had an unelongated morphology. At the end of the experiment time, both *C. albicans* strains, which belonged to the treated groups (*C. albicans* with pre-coating aptamer), showed a similar phenotype response to either CCSC or NCSC exposure. Our result contradicted a previous report that demonstrated

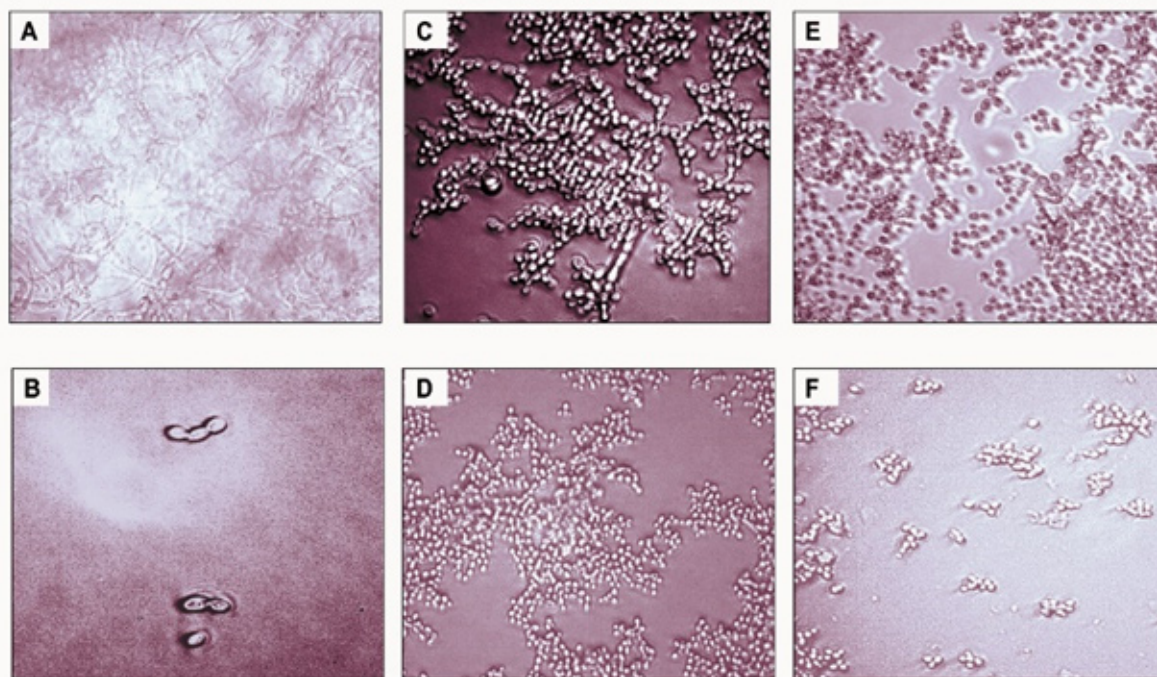


Figure 5. Light microscopy photomicrograph illustrating the different morphology of *C. albicans* with and without pre-coating aptamer (Ca-apt1) and grown for 48-h in the growth medium supplemented with cigarette smoke condensate (CSC). The pictures show biofilm of *C. albicans* derived from clinical isolate, without either pre-coating aptamer or added CSC (A), *C. albicans* with pre-coated aptamer but untreated with either CSC/ CCSC or NCSC (B), *C. albicans* pre-coated with 1 ng/μL aptamer, CCSC (C) and NCSC (E), and 10% aptamer with CCSC (D) and NCSC (F). All microscopic image show at X 200 magnification.

that the clinical isolate showed less biofilm growth activity than the laboratory strain⁴⁰. We reasoned that the similar trend found in either *C. albicans* strain used in this study is probably because the clinical isolate and the ATCC strain used in this study have a similar karyotype as it has been previously reported⁴¹.

Conclusion

Regardless of the mechanism involved, this study demonstrated that both CCSC and NCSC have a potential to enhance biofilm formation by *C. albicans*. By exposing either cigarette smoke prepared from clove and non-clove cigarettes, *C. albicans*, either clinical isolate or the reference strain, shows a similar trend in its capacity to form biofilm. However, the pattern of biofilm formation was different. Besides, this study clearly indicates that the aptamer (Ca-apt1) had an impact on the degree of cell adhesion and its concentration affected the profile of biofilm formation at the maturity phase. However, the underlying mechanisms remain to be investigated at molecular level. The

underlying mechanism is particularly important for pathogenic fungus, such as *C. albicans*, during exposure to tobacco smoke.

Data availability

Underlying data

Figshare: Underlying data for 'The discrepancy between Clove and Non-Clove Cigarette Smoke-Promoted *Candida albicans* Biofilm Formation with pre-coating RNA-aptamer', DOI 10.17605/OSF.IO/WF2KB⁴²

This project contains the following underlying data:

- Data for Figs 2-4 CCSC-2021 F1000 Aptamer Cigarette.xlsx

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Version 3

Reviewer Report 23 August 2021

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Zamirah Zainal-Abidin 

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There are still some comments from the second review yet to be addressed by the authors:

1. Abstract: In the sentence "Interestingly, we noted that the enhancer effect of treated CCSC..." the word 'treated' is still not replaced with 'added' since the CCSC was not treated in this study.
2. Introduction: In paragraph 3, the word 'strain' should be replaced with 'strand'. The term 'strain' is not used to describe DNA and RNA.
3. Methods: In the final sentence of the subheading 'Preparation of cigarette smoke condensates', please specify what is being 'kept at 4°C until use.'

I hope the authors will address the above comments. Thank you.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacology, Oral Microbiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 22 July 2021

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**Samira Shirooie**

Pharmaceutical Sciences Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran

I have checked the revised article and the author's responses. In my opinion, the revisions are appropriate.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 13 July 2021

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**Zamirah Zainal-Abidin** 

Department of Craniofacial Diagnostics and Biosciences, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia

Almost all comments and suggestions submitted during the initial review were addressed.

There are a few minor corrections as follows:

1. Abstract: the word 'treated' is still not replaced with 'added'.
2. Introduction: In paragraph 3, the word 'strain' should be replaced with 'strand'.
3. Methods: In the final sentence, please specify what is being 'kept at 4°C until use.'

That is all, thank you.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacology, Oral Microbiology

I confirm that I have read this submission and believe that I have an appropriate level of

expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 30 June 2021

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Zamirah Zainal-Abidin 

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This article reported a study on the effects of two types of cigarette smoke condensates (CSC), namely the clove CSC (CCSC) and non-clove CSC (NCSC) on the formation of biofilm by *C. albicans* from clinical isolate and ATCC 10231. This study also investigated the effects of pre-coating RNA aptamer, Ca-apt1 in modulating the gene expression and phenotypic changes of *C. albicans* cells that have been exposed to the abovementioned types of SCC, with the result of modulation is concentration-dependent.

The authors found that *C. albicans* cells of both clinical isolates and ATCC strains, precoated with different concentrations (1 ng/μL and 10 ng/μL) of aptamer (Ca-apt1) showed downregulation of adhesion-associated genes, i.e., *ALS3* and *HWP1*, where the relative fold changes of the expression of the studied genes were Ca-apt1 concentration-dependent. This finding was substantiated by a microscopic study of the formation of the biofilm by *C. albicans*, compared with the ones without Ca-apt1.

The same approach of the investigation was adopted to investigate whether Ca-apt1 confers a protective effect on the CCSC and NCSC induced biofilm formation by *C. albicans*. The results demonstrated that CCS-induced biofilm formation was mitigated in the presence of Ca-apt1. The authors proposed that Ca-apt1 modulates the morphogenesis of *C. albicans* growing as a biofilm, as indicated by the predominance of blastospores in the treated Ca-apt1 biofilm, relative to the untreated control.

Since adhesion is the crucial step in biofilm formation, the authors suggested that the C-apt1 may bind to a common chemical structure of the *C. albicans* cells that may play a vital role in the adherence of the fungus to a substrate. This suggestion is indeed intriguing, which warrants further studies on the anti-adhesion mechanism of Ca-apt1.

The most specific comments based on the sections in the manuscript are as follows:

Title:

Capitalization of the title needs to be consistent.

Abstract:

In the sentence "Interestingly, we noted that the enhancer effect of treated CCSC was no longer effective...". As described in the Methods section, the CCSC did not undergo any form of treatment in this study. It is recommended to rephrase this sentence to present the actual form of the CCSC used in this study.

Results:

1. The first subheading should be reporting on *ALS3* and *HWP1* instead of *HWP1* and *YWP1*.
2. For Figures 2A and B, it is recommended that the Y-axes be standardized in both graphs, with a maximum relative fold change = 1.0. The comparison between the two strains in terms of inhibition of biofilm formation may then be emphasized. The indication of the dual asterisk (**) in the graphs should also be described. In the subtitle, use 1 ng/ μ L and 10 ng/ μ L rather than 1% and 10%. The authors may also describe Figure 2C-E in terms of comparison to the abundance of adherent *C. albicans* cells attached to the surface between the 3 treatments.
3. In Figure 4, it is recommended that the Y-axes are standardized in both graphs, with a maximum percentage of control = 100. The comparison between the two strains in terms of reduction of the pre-formed biofilm can then be highlighted.

Discussion:

1. In paragraph 4, in this study, the comparison in terms of chemical contents between NCSC and CCSC is not reported. The concentrations of the common chemical compounds may be different among cigarette manufacturers. Therefore, it is recommended to provide relevant information on the concentrations of CSC chemicals in cigarettes used specifically for this study.
2. In the final paragraph, the statement "Hence, it affirms that the anti-adhesion effect of the aptamer on biofilm development is enhanced by conventional cigarette smoke". The authors should point out the results in this study or cite references that compared the formation of *C. albicans* biofilms without CSC to support that statement.
3. In the statement "...with the minimum concentration (1 ng/ μ L) of CCSC...", it is supposed to be aptamer.

Conclusion:

In the statement "...*C. albicans*, either clinical isolate or the reference strain, shows a similar trend." The authors will need to elaborate a little more on what trend they are referring to e.g., gene expression?

Other comments:

The manuscript is well written, with minor grammatical errors and some typos, which can be easily corrected by giving it a manuscript editing round-up.

In general, the authors have shown in this study the potential of Ca-apt1, an RNA aptamer in modulating virulence factors of *C. albicans*, one of the opportunist microorganisms within the oral cavity. In addition, the authors also have shown that cigarette smoke condensates can exacerbate

the growth of *C. albicans* biofilms and how adding Ca-apt1 has mitigated this effect. I hope the authors will further investigate the underlying protective mechanism of aptamer at both genotypic and phenotypic levels, against the exacerbation of biofilm formation and pathogenicity of *C. albicans* when they are exposed to cigarette smoke, as well as adopt a tissue culture or animal model appropriate for investigation.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacology, Oral Microbiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 03 Jul 2021

Boy Muchlis Bachtiar, Faculty of Dentistry Universitas Indonesia, Jakarta, Indonesia

Reviewer # 2

The most specific comments based on the sections in the manuscript are as follows:

Title:

Capitalization of the title needs to be consistent.

Author's response (AR): We agree. Thank you. As suggested, we have corrected the capitalization of the title.

Abstract:

In the sentence "Interestingly, we noted that the enhancer effect of treated CCSC was no longer effective...". As described in the Methods section, the CCSC did not undergo any form of treatment in this study. It is recommended to rephrase this sentence to present the

actual form of the CCSC used in this study.

AR: Thank you for this important correction. Hence, we changed the word to make clear the meaning, as follows;

"...we noted that the enhancer effect of added CCSC was no longer effective when the yeast had been precoated with 10 ng/ μ L aptamer tested".

3. Results:

3.1. The first subheading should be reporting on *ALS3* and *HWP1* instead of *HWP1* and *YWP1*.

AR: Yes, you right. Thank you. We have changed the name of the gene, as suggested.

3.2. For Figures 2A and B, it is recommended that the Y-axes be standardized in both graphs, with a maximum relative fold change = 1.0. The comparison between the two strains in terms of inhibition of biofilm formation may then be emphasized. The indication of the dual asterisk (**) in the graphs should also be described. In the subtitle, use 1 ng/ μ L and 10 ng/ μ L rather than 1% and 10%. The authors may also describe Figure 2C-E in terms of comparison to the abundance of adherent *C. albicans* cells attached to the surface between the 3 treatments.

AR: As suggested by the reviewer, we have standardized the Y-axes in both graphs, where the maximum relative fold change has been set to 1. Moreover, as corrected by the reviewer#1, the dual asterisks were removed. The typos in the subtitle, 1 and 10%, has been changed to 1 and 10 ng/ μ L. Lastly, we described the figure 2C-E, where the abundance of adherent *C. albicans* between the 3 treatments was compared. Thank you.

3.3. In Figure 4, it is recommended that the Y-axes are standardized in both graphs, with a maximum percentage of control = 100. The comparison between the two strains in terms of reduction of the pre-formed biofilm can then be highlighted.

AR: yes. We agree. Accordingly, we changed the T-axes in both graphs with a maximum percentage of the control set at 100. Thank you.

4. Discussion:

4.1. In paragraph 4, in this study, the comparison in terms of chemical contents between NCSC and CCSC is not reported. The concentrations of the common chemical compounds may be different among cigarette manufacturers. Therefore, it is recommended to provide relevant information on the concentrations of CSC chemicals in cigarettes used specifically for this study.

AR: Thank you for the recommendation, and we agree. Thus, as suggested, in paragraph 4, we added a relevant information regarding the smoke chemical in CCSC and NCSC used in this study.

4.2. In the final paragraph, the statement "Hence, it affirms that the anti-adhesion effect of the aptamer on biofilm development is enhanced by conventional cigarette smoke". The authors should point out the results in this study or cite references that compared the formation of *C. albicans* biofilms without CSC to support that statement.

AR: We agree, and we have revised the sentence, and added references needed. It can be seen in the revised version of this manuscript; the revised sentence as follows: "Hence, it affirms that the anti-adhesion effect of the aptamer on biofilm development is enhanced accelerated by conventional cigarette smoke^{5, 7}." Thank you.

4.3. In the statement "...with the minimum concentration (1 ng/μL) of CCSC...", it is supposed to be aptamer.

AR: We agree, and we have corrected the typo. Thank you.

5. Conclusion:

In the statement "...*C. albicans*, either clinical isolate or the reference strain, shows a similar trend." The authors will need to elaborate a little more on what trend they are referring to e.g., gene expression?

AR: We agree. Thus, we revised the sentence to elaborate the meaning of "trend" specifically. Hence, the new sentence is now becoming : "...shows a similar trend in its capacity to form biofilm."

6. Other comments:

The manuscript is well written, with minor grammatical errors and some typos, which can be easily corrected by giving it a manuscript editing round-up. In general, the authors have shown in this study the potential of Ca-apt1, an RNA aptamer in modulating virulence factors of *C. albicans*, one of the opportunist microorganisms within the oral cavity. In addition, the authors also have shown that cigarette smoke condensates can exacerbate the growth of *C. albicans* biofilms and how adding Ca-apt1 has mitigated this effect. I hope the authors will further investigate the underlying protective mechanism of aptamer at both genotypic and phenotypic levels, against the exacerbation of biofilm formation and pathogenicity of *C. albicans* when they are exposed to cigarette smoke, as well as adopt a tissue culture or animal model appropriate for investigation.

AR: Thank you. Hence, we added the reviewer's suggestion at the end of the conclusion.

Competing Interests: No competing interest

Reviewer Report 29 June 2021

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Samira Shirooie

Pharmaceutical Sciences Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran

In the introduction, mention the genes ALS3 & HWP1 and their roles.

In results, in the first headline was written "Evaluation of the mRNA levels of *HWP1* and **YWPI** in pre-coating aptamer *C. albicans*" that must be corrected to "Evaluation of the mRNA levels of *HWP1* and **ALS3** in pre-coating aptamer *C. albicans*".

The data of the control group should be shown in figures 2A and 2B.

Write abbreviations in separate part before introduction.

In figures 3 A & B, compare the CCSC and NCSC groups with their control group not with the another control group, for example compare CCSC for HWP1 expression level with the control group of HWP1 not with ALS3.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 03 Jul 2021

Boy Muchlis Bachtiar, Faculty of Dentistry Universitas Indonesia, Jakarta, Indonesia

Reviewer #1

1. In the introduction, mention the genes ALS3 & HWP1 and their roles.

Author's response (**AR**): We have mentioned the suggestion, and it can be seen at the end of paragraph one of the introduction section, as follows;

...In this regard, the presence of nicotine in cigarette smoke promotes C. albicans growth rate and adhesion, in which, a higher level of expression of genes related to adherence, such as ALS3 and HWP1 have been reported⁹.

2. In results, in the first headline was written "Evaluation of the mRNA levels of *HWP1* and *YWP1* in pre-coating aptamer *C. albicans*" that must be corrected to "Evaluation of the mRNA levels of *HWP1* and **ALS3** in pre-coating aptamer *C. albicans*."

AR: Yes, you right. Thank you. Accordingly, we have corrected the name of the gene.

3. The data of the control group should be shown in figures 2A and 2B.

AR: As suggested, the control groups have been added in figures 2A and 2B. Thank you.

4. Write abbreviations in separate part before introduction.

AR: We only use abbreviations or acronym for they have been widely known in scientific or academic communication.

5. In figures 3 A & B, compare the CCSC and NCSC groups with their control group not with another control group, for example compare CCSC for HWP1 expression level with the control group of HWP1 not with ALS3.

AR: Thank you for this important suggestion. As suggested, we have deleted the double asterisks, as it can be confusing. Thank you.

Competing Interests: No competing interests

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